A modified pyrroloquinoline quinone-dependent glucose dehydrogenase having a low reactivity with respect to maltose, galactose, etc. is provided. A modified pyrroloquinoline quinone-dependent glucose dehydrogenase including an amino acid sequence in which one or more amino acids in a region corresponding to a first region consisting of amino acids at positions 326 to 354 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are substituted as compared with an amino acid sequence of the corresponding wild-type enzyme.

5 Claims, 6 Drawing Sheets
The sequence shown in Fig. 2 is:

```
ATGAATAAATACTTTATTGGCTAAAAATTACTTTATTAGGGCCTGCTCAGTACTCTACGCTCAATTACAT
TTGCTGATGTTCCCTACACACACTCCTACATTGTGAAGAGAAAAGCAGGGGCTGATCTCTGTGAAAACCTAGAG
CGGGCAACAGGAAGAGTCTAACCAAGTAGACTCAGGCGACATCTGAAACATCTAGACAGCTCTCATATACAG
AATATCTTATATATCTTATTCTGAGATCTCTTATTTATCCAAATCTCAAGAAGATACCCAGGAAATG
AGATTTAAACTGTGCGATATTCAAAAACCGTAAACGTTACGCTGTCAGGCCACAGCCACAAAGATTTACT
ATATCGATGTTGATCAGGGCGTTAACAGCGCTGCTTATTCTTACAAAATCGAAATTTTTCTTAAATCCAA
CGACTCAACAGGAACTGAGCGCCAAGAAGACTACATACTCACTATATGTTAAAGCTATTACGCTTAAATCTG
GATGGAAGATTTTAAAAGATTACAGTTAAAGTTGAATTACGATTTTCTCAGATGCTGTCATCTC
GTAATCCACAGGCGTTCTGAGCTTATGCTCAAATGTTAAAGGTGCTAAACGCGGATACGTTGAAATCTCT
AGGATGAATATTCTGTTGCTTTGCGAGATGTTGGGCTGCTTATTCTGTTCTTTGCTGCTTATTCTGTTCTT
AAAAGGAGGAAATGAGGGAAGGAAATGGGAGGAAATGAGGGAAGGAAATGGGAGGAAATGAGGGAAGGAAATG
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<td>S-primer (G)-</td>
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<td>0.017</td>
</tr>
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<td>10-SF</td>
<td>0.2*(0.5/3)*0.5=</td>
<td>0.017</td>
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<tr>
<td></td>
<td></td>
<td>10-GF</td>
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</tr>
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<tr>
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<td>0.067</td>
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<td>10-SL</td>
<td>0.8*(0.5/3)*0.5=</td>
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<td>0.8*(0.5/3)*0.5=</td>
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<td>9-SF</td>
<td>0.2*(0.5/3)*0.5=</td>
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<td>0.2*(0.5/3)*0.5=</td>
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<td>0.2<em>0.5</em>0.5=</td>
<td>0.050</td>
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<td>0.2*(0.5/3)*0.5=</td>
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<tr>
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<td>9-SL</td>
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<td>9-QF</td>
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<td>0.200</td>
</tr>
<tr>
<td></td>
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<td>0.8*(0.5/3)*0.5=</td>
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<td>0.200</td>
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Fig. 6

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FIELD OF THE INVENTION

The present invention relates to a pyrroloquinoline quinone-dependent glucose dehydrogenase. More particularly, the present invention relates to a modified pyrroloquinoline quinone-dependent glucose dehydrogenase in which an amino acid in a certain region is substituted by another amino acid as compared with a wild-type enzyme. The modified pyrroloquinoline quinone-dependent glucose dehydrogenase of the present invention is used for measurement of an amount of glucose in, for example, a clinical test.

BACKGROUND OF THE INVENTION

A pyrroloquinoline quinone-dependent β-D-glucose dehydrogenase (EC1.1.1.199, PQQGDH) is conjugated with a coenzyme, pyrrolo-quinoline quinone (PQQ) to catalyze a reaction of oxidizing β-D-glucose to produce gluconolactone. With this property, PQQGDH is used for determining an amount of glucose in a clinical test, a food analysis, monitoring of a culture process, and the like.

As examples of PQGQDH reported in the past, a PQGQDH produced by Acinetobacter species L.M.D.79.41 strain and a modified PQGQDH thereof are known (for example, JP 2000-312588A, JP 2000-350588A, JP 2000-197888A, JP 2001-346587A, WO02-34919 (A1), and A-M. Cleaton-Jansen et al., Mol. Gen. Genet., 217, 430 (1989)). The substrate specificity of the PQGQDH produced by L.M.D.79.41 strain is low. For example, also with respect to maltose, the reactivity of the PQGQDH has a reactivity corresponding to about 90% of the reactivity with respect to glucose.

SUMMARY OF THE INVENTION

In order to measure an exact amount of glucose, a PQGQDH is required to have high substrate specificity. For example, when carrying out diagnosis of diabetes for patients who undergo drip transfusion of blood, if PQGQDH having low substrate specificity is used, the obtained measurement value includes the amount of maltose in a transfusion liquid in addition to the amount of glucose. Thus, the exact amount of glucose in blood cannot be determined.

Similarly, for example, when carrying out measurement for patients with hepatic disorder, if PQGQDH having low substrate specificity is used, due to the influence of galactose, reliable measurement may not be carried out. In this way, since PQGQDH having low substrate specificity is much influenced by other sugars such as maltose, an exact amount of glucose cannot be measured.

In the above-mentioned modified PQGQDH reported in the past, by substituting a part of amino acids of the wild-type PQGQDH, the substrate specificity is improved. However, reactivity with respect to maltose or galactose is still considerably remained. Therefore, further improvement of the substrate specificity for enabling the exact measurement of an amount of glucose has been desired. In particular, PQGQDH with sufficiently low reactivity with respect to galactose has not been found and been desired to be provided.

Under such circumstances, it is an object of the present invention to provide a modified PQGQDH having high substrate specificity with respect to glucose. In particular, it is an object of the present invention to provide a modified PQGQDH with extremely low reactivity with respect to galactose in addition to low reactivity with respect to maltose. Furthermore, the present invention also aims to provide a modified PQGQDH using the polynucleotide, expression vector and a transformant, which can be used for preparing the modified PQGQDH, and a method for preparing the modified PQGQDH using the polynucleotide, expression vector and transformant, and a kit for measuring glucose.

In order to achieve the above-mentioned objects, the present inventors have investigated in detail the relation between the substitution of amino acids in a certain region and the change in the substrate specificity in PQGQDH produced by Acinetobacter calcoaceticus. As a result, the present inventors succeeded in finding a plurality of regions deeply involved in the substrate specificity. That is to say, as mentioned in the below-mentioned Examples, the present inventors found that when the amino acid at position 351 was substituted in a wild-type PQGQDH of Acinetobacter calcoaceticus, the reactivity with respect to maltose and galactose was significantly lowered. Furthermore, the present inventors found that the amino acid at position 342 was also deeply involved in the substrate specificity. Herein, since these amino acids were located in a secondary structure including amino acid residues at positions 326 to 354, it was thought that by modifying this structural part, the substrate specificity with respect to glucose can be efficiently improved.

Furthermore, an amino acid residue at position 295 located in the secondary structure including amino acid residues at positions 278 to 320 was shown to be largely involved in the reactivity with respect to maltose and galactose. Thus, it was thought that the modification of this structural part was also effective for improving the specificity. Similarly, since it was observed that the substitution of the amino acid residue at position 169 also improved the substrate specificity, it was thought that the modification of the secondary structural part including amino acid residues at positions 162 to 197 was effective in improving the substrate specificity.

Furthermore, it was suggested that some other amino acid residues were involved in the substrate specificity. Note here that the present inventors succeeded in obtaining various modified PQGQDHs in which amino acids were substituted in at least two or more positions 75, 168, 169, 295, 342, 347 and 351. In these modified PQGQDHs, the substrate specificity with respect to glucose was observed to be radically improved. In particular, it was clarified that in the modified PQGQDH in which amino acids at positions 169, 295, 342 and 351 were substituted had extremely low reactivity with respect to maltose and galactose. Similarly, it was clarified that the modified PQGQDH in which the amino acid at positions 342 and 351 were substituted also have extremely low reactivity with respect to maltose and galactose.

Herein, proteins having the same functions are generally similar to each other in structure of the region involved in its activity site or substrate specificity. Therefore, it is expected that the above-mentioned information as to the relation between the substitution of a certain amino acids and the substrate specificity in PQGQDH derived from Acinetobacter calcoaceticus may be applied to PQGQDH derived from other microorganisms.

The present invention was completed based on the above-mentioned findings and provides the following configurations.
[1] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising an amino acid sequence in which one or more amino acids in a region corresponding to a first region consisting of amino acids at positions 326 to 354 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are substituted as compared with an amino acid sequence of the corresponding wild-type enzyme.

[2] The modified pyrroloquinoline quinone-dependent glucose dehydrogenase described in [1], wherein in said region corresponding to the first region, two or more amino acids including amino acids corresponding to amino acids at positions 342 and 351 are substituted.

[3] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising an amino acid sequence in which in a first region consisting of amino acids at positions 326 to 354 in the amino acid sequence of SEQ ID NO: 1, one or more amino acids are substituted by other amino acids.

[4] The modified pyrroloquinoline quinone-dependent glucose dehydrogenase described in [3], wherein in said first region, two or more amino acids including amino acids at positions 342 and 351 are substituted by other amino acids.

[5] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising an amino acid sequence in which an amino acid corresponding to an amino acid at position 295 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus is substituted by another amino acid as compared with an amino acid sequence of the corresponding wild-type enzyme.

[6] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising an amino acid sequence in which amino acids corresponding to amino acids of the following (1) and (2) in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are respectively substituted by other amino acids as compared with an amino acid sequence of the corresponding wild-type enzyme:

(1) an amino acid at position 295; and
(2) one or more amino acids selected from the group consisting of amino acids at positions 75, 168, 169, 342, 347 and 351.

[7] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising any of the following amino acid sequences (1) to (6):

(1) an amino acid sequence in which amino acids corresponding to amino acids at positions 169, 295, 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;

(2) an amino acid sequence in which amino acids corresponding to amino acids at positions 295 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;

(3) an amino acid sequence in which amino acids corresponding to amino acids at positions 295, 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;

(4) an amino acid sequence in which amino acids corresponding to amino acids at positions 169, 295 and 342 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;

(5) an amino acid sequence in which amino acids corresponding to amino acids at positions 75, 295, 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme; and

(6) an amino acid sequence in which amino acids corresponding to amino acids at positions 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from Acinetobacter calcoaceticus are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme.

[8] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising any of the following amino acid sequences (1) to (6):

(1) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 169, 295, 342 and 351 are substituted by other amino acids respectively;

(2) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 295 and 351 are substituted by other amino acids respectively;

(3) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 295 and 351 are substituted by other amino acids respectively;

(4) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 169, 295 and 342 are substituted by other amino acids respectively;

(5) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 75, 295, 342 and 351 are substituted by other amino acids respectively; and

(6) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 342 and 351 are substituted by other amino acids respectively.

[9] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising any of amino acid sequences of SEQ ID NOs: 28 to 34.

[10] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, wherein the reactivity with respect to maltose is 40% or less and the reactivity with respect to galactose is 10% or less, respectively relative to the reactivity with respect to glucose.

[11] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, wherein the reactivity with respect to maltose is 20% or less and the reactivity with respect to galactose is 5% or less, respectively relative to the reactivity with respect to glucose.

[12] A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, wherein the reactivity with respect to galactose is 10% or less relative to the reactivity with respect to glucose.


[14] A vector containing polynucleotide described [13].


[16] A method for producing pyrroloquinoline quinone-dependent glucose dehydrogenase, the method comprising
a step of culturing the transformant described in [15] in a state in which the polynucleotide can be expressed; and a step of separating the expressed product of the polynucleotide.

[17] A kit for measuring glucose, comprising the modified pyrrolquinoline quinone-dependent glucose dehydrogenase described in any of [1] to [12].

The modified pyrrolquinoline quinone-dependent glucose dehydrogenase provided in the present invention has high specificity with respect to glucose. Therefore, by using such modified enzyme, an amount of glucose can be determined with high accuracy.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects and technical advantages of the present invention will be readily apparent from the following description of the preferred exemplary embodiments of the invention in conjunction with the accompanying drawings, in which:

FIG. 1 shows a sequence (not including the signal region) of a gene encoding pyrrolquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* and an amino acid sequence (not including the signal region).

FIG. 2 shows a sequence (including the signal region) of a gene encoding pyrrolquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* and an amino acid sequence (including the signal region).

FIG. 3 shows a structure of an expression vector pUKGDHB(S) used in Examples.

FIG. 4 is a table showing primers used in Mutation scrambling and the amplification sites.

FIG. 5 is a table showing a mixing ratio of each DNA fragment in Mutation scrambling. The setting bias rates are made to be 0.8 for G75, G295, M342 and 351, and 0.5 for Q168, L169 and W347, wherein the setting bias rate means a rate of amino acid substitution introduced by mutation scrambling.

FIG. 6 is a table showing positions of amino acid substitution and substrate specificity in various kinds of modified amino acid substitution obtained by mutation scrambling in Examples. Reactivities with respect to various substrates are shown by relative values when the reactivity with respect to glucose is represented by a value (100).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a modified PQQGDH in which an amino acid sequence in a certain region is substituted as compared with the corresponding wild-type enzyme. The modified PQQGDH of the present invention is typically prepared or designed by substituting a part of amino acids by another amino acids in naturally occurring PQQGDH, i.e., a wild-type PQQGDH. In this way, the wild-type PQQGDH which the modified PQQGDH of the present invention is based on (derived from) is herein denoted by “the corresponding wild-type enzyme.” Examples of the wild-type enzyme of the present invention include a PQQGDH produced by oxidizing bacteria such as *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Burkholderia cepacia*, *Glucobacter oxydans*, *Acetobacter aceti*, etc., and *Agrobacterium radiobacter* *Escherichia coli*, *Klebsiella aerogenes*, and the like.

The term “corresponding” used to explain an amino acid residue or region means that between proteins (enzymes) to be compared, the functions resulting from the structure are equal to each other, and particularly means that the functions in terms of the reactivity with respect to sugars other than glucose, for example, maltose, galactose, are equal. For example, in PQQGDH derived from microorganisms other than *Acinetobacter calcoaceticus*, when the primary structures of the PQQGDH derived from the other microorganisms is compared with that of the PQQGDH derived from *Acinetobacter calcoaceticus* and the homology in the secondary structures of both PQQGDHs are taken into consideration, a region of the PQQGDH derived from the other microorganisms which is reasonably thought to have the same function as one of the secondary structure regions of PQQGDH derived from *Acinetobacter calcoaceticus* can be referred to as “corresponding to the secondary structure region of PQQGDH derived from *Acinetobacter calcoaceticus*.”

Positions of the amino acids in the present specification are specified by reference numbers sequentially given from the side of the N-terminal toward the C-terminal with aspartic acid at N-terminal given the reference number 1.

In one embodiment of the modified PQQGDH according to the present invention, as compared with the amino acid sequence of the corresponding wild-type enzyme, one or more amino acids are substituted by other amino acids in a region corresponding to a first region consisting of amino acids at positions 326 to 354 in pyrrolquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* (hereinafter, referred to as “A. C. PQQGDH”). The amino acid sequence of A. C. PQQGDH in the present application is a sequence specified by SEQ ID NO: 1. This sequence is an amino acid sequence of PQQGDH produced by *Acinetobacter calcoaceticus* IFO 12552 strain as mentioned in Examples below.

In another embodiment of the modified PQQGDH of the present invention, instead of or in addition to the above-mentioned amino acid substitution, one or more amino acids are substituted by other amino acids in a region corresponding to a second region consisting of amino acids at positions 278 to 320 in A. C. PQQGDH.

Furthermore, in a further embodiment of the modified PQQGDH, instead of or in addition to the above-mentioned amino acid substitution, in a region corresponding to a third region consisting of amino acids at positions 162 to 197 in A. C. PQQGDH, one or more amino acids are substituted by other amino acids.

Furthermore, in a further embodiment of the modified PQQGDH, instead of or in addition to the above-mentioned amino acid substitution, from the group consisting of amino acids which respectively correspond to amino acids at positions 75, 168 to 347 in A. C. PQQGDH are substituted by other amino acids.

The number of amino acids to be substituted in each region of the present invention is not specifically limited. However, it is preferable that two or more amino acids are substituted in the case of the region corresponding to the first region.

Furthermore, the position of amino acids to be substituted in each region is not particularly limited. For example, in the region corresponding to the first region, it is preferable that amino acids corresponding to the amino acids at positions 342 and/or 351 in the first region are substituted. Similarly, in the region corresponding to the second region, it is preferable that amino acids corresponding to the amino acid at position 295 in the second region is substituted; and in the region corresponding to the third region, it is preferable that
amino acids corresponding to the amino acids at position 75 and/or 169 in the third region are substituted.

Herein, the kinds of the amino acids after substitution are not particularly limited. For example, the amino acid at position 75 is tryptophan, the amino acid at position 168 is histidine, the amino acid at position 169 is phenylalanine, the amino acid at position 295 includes glutamic acid, aspartic acid, tyrosine, phenylalanine, etc., the amino acid at position 342 is proline, the amino acid at position 347 is arginine, and the amino acid at position 351 is threonine.

Specific examples of the modified PQQGDH of the present invention include PQQGDH having an amino acid sequence of any of SEQ ID Nos: 28 to 34. Herein, the amino acid sequence of SEQ ID NO: 28 is an amino acid sequence in which amino acids at positions 169, 295, 342 and 351 are substituted by other amino acids respectively in the amino acid sequence (SEQ ID NO: 1) of the wild-type PQQGDH produced by Acinetobacter calcoaceticus IFO 12552 strain. Similarly, the amino acid sequence of SEQ ID NO: 29 is an amino acid sequence in which amino acids at positions 169, 295, 342 and 351 are substituted by other amino acids respectively in SEQ ID NO: 1. Similarly, the amino acid sequence of SEQ ID NO: 30 is an amino acid sequence in which amino acids at positions 169, 295, 342 and 351 are substituted by other amino acids respectively in SEQ ID NO: 1. Similarly, the amino acid sequence of SEQ ID NO: 31 is an amino acid sequence in which amino acids at positions 295, 342 and 351 are substituted by other amino acids respectively in SEQ ID NO: 1. Similarly, the amino acid sequence of SEQ ID NO: 32 is an amino acid sequence in which amino acids at positions 169, 295 and 342 are substituted by other amino acids respectively in SEQ ID NO: 1. Similarly, the amino acid sequence of SEQ ID NO: 33 is an amino acid sequence in which amino acids at positions 169, 295, 342 and 351 are substituted by other amino acids respectively in SEQ ID NO: 1. Similarly, the amino acid sequence of SEQ ID NO: 34 is an amino acid sequence in which amino acids at positions 342 and 351 are substituted by other amino acids respectively in SEQ ID NO: 1.

As shown in the below-mentioned Examples, in the modified PQQGDH having an amino acid sequence of SEQ ID NO: 28 or an amino acid sequence of SEQ ID NO: 34, the reactivity with respect to maltose is extremely low. Furthermore, in the modified PQQGDH having an amino acid sequence of SEQ ID NO: 28, an amino acid sequence of SEQ ID NO: 29, an amino acid sequence of SEQ ID NO: 33 or an amino acid sequence of SEQ ID NO: 34, the reactivity with respect to galactose is extremely low.

The modified PQQGDH of the present invention has a feature that the selectivity with respect to glucose is improved as compared with the wild type enzyme. That is to say, the modified PQQGDH of the present invention has a feature that the reactivity with respect to maltose or lactose is low and in particular the reactivity with respect to galactose is significantly low as compared with the wild type enzyme. When the reactivity is expressed by relative values respectively relative to 100% (the reactivity with respect to glucose), the reactivity of the modified PQQGDH of the present invention with respect to maltose is preferably 40% or less, more preferably 30% or less and furthermore, preferably 20% or less. Similarly, the reactivity with respect to lactose is preferably 50% or less and more preferably 40% or less. The reactivity with respect to galactose is preferably 20% or less, more preferably 10% or less, furthermore, preferably 5% or less and the most preferably substantially 0%. Note here that just “substrate specificity” herein denotes the selectivity with respect to glucose.

In the modified PQQGDH of the present invention, in addition to the above-mentioned amino acid substitution, a part of amino acids may be further modified. The phrase “a part of amino acids is modified” herein is intended to include deletion or substitution of one to several amino acids constituting amino acid sequence, or addition or insertion of one to several amino acids, or the combination thereof so as to change the amino acid sequence. In principle, such modification can be carried out as long as the activity as the PQQGDH (i.e., reactivity with respect to glucose) is maintained. However, even though the activity of PQQGDH is lowered to some degree, in a case where the reactivity with respect to other substrate (for example, maltose or galactose) is also lowered, so that the substrate specificity with respect to glucose is turned out to be improved, or in a case where lowering of the substrate specificity to some degree does not influence on the measurement of an amount of glucose, modification as mentioned above are allowed.

It is preferable that the modification of a part of the amino acids as mentioned above is carried out in a state in which the reactivity with respect to maltose, galactose and the like is kept low.

Herein, the position of the amino acid to be modified is not particularly limited. Furthermore, modification may be carried out in a plurality of positions. “Plurality of positions” herein denotes 10% or less of the number of whole amino acids, and preferably 5% or less. Furthermore preferably, it is 1% or less of the number of whole amino acids.

The modified PQQGDH of the present invention can be also prepared by firstly obtaining gene encoding a wild-type PQQGDH; then modifying the wild-type PQQGDH so as to construct polynucleotide encoding the modified PQQGDH; and finally expressing the polynucleotide in an appropriate expression system. Hereinafter, this preparing method will be described. Note here that once a sequence is designed, based on its sequence information, polynucleotide (gene) encoding the modified PQQGDH of the present invention can be prepared by chemical synthesis using deoxyribonucleotide triphosphoric acids (dATP, dTTP, dGTP and dCTP).

<Obtaining Gene Encoding Wild-Type PQQGDH>

Firstly, a gene encoding PQQGDH is obtained from bacteria producing PQQGDH. Examples of the bacteria producing PQQGDH include oxidizing bacteria such as Acinetobacter calcoaceticus, Acinetobacter baumannii, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas fluorescens, Burkholderia cepacia, Gluconobacter oxydans, Acetobacter acetii, etc.; and enterobacteria such as Escherichia coli, Klebsiella pneumoniae, etc. Above all, it is preferable that Acinetobacter calcoaceticus is used as bacteria producing PQQGDH.

A gene encoding PQQGDH can be extracted from the cell body of the bacteria producing PQQGDH in the usual manner. A PQQGDH gene can also be prepared by employing PCR method, etc. using the genome DNA of PQQGDH as a template. On the other hand, an extracted gene is amplified by using an amplification method such as PCR method, and then the amplification product may be used in the following operations. Note here that after the base sequence of the gene encoding PQQGDH is identified, the targeted PQQGDH gene can be prepared also by a chemical technique. Hereinafter, as one example of preparing a PQQGDH gene, a case where the preparation using Acinetobacter calcoaceticus IFO 12552 strain as a starting material will be described in detail.

Firstly, a DNA fragment obtained by isolating and purifying a chromosome of Acinetobacter calcoaceticus IFO
12552 and thereafter subjecting it to an ultrasonication treatment, a restriction enzyme treatment, etc. and an expression vector prepared in a linear state are ligated to each other at their both blunt ends or sticky ends so as to be closed with the use of DNA ligase, etc. Thus, a recombinant vector is constructed. A recombinant vector can be also constructed by amplifying the POQGDH gene by a polymerase chain reaction (PCR) using an oligonucleotide primer designed based on the base sequence of the well-known POQGDH derived from Acinetobacter species L.M.D 79.41 strain and ligating thereof with the use of DNA ligase. Then, the recombinant vector that was constructed by any of the above-mentioned methods is transferred into a host microorganism in which the vector can autonomously replicate.

Then, screening is carried out using a marker unique to an expression vector and POQGDH activity as indices, and thus a host microorganism transformed by a recombinant vector containing a gene encoding POQGDH is obtained.

After the selected transformant is cultured if necessary, cell body is collected. A recombinant vector is isolated and purified from the collected cell body in the usual manner. Thus, a recombinant vector containing the targeted POQGDH gene is obtained. The isolation of the POQGDH gene from the recombinant vector can be carried out by restriction enzyme treatment, etc.

Herein, one example of obtaining POQGDH gene from Acinetobacter calcoaceticus IFO 12552 is described in detail. First of all, a culture medium, which is obtained by culturing Acinetobacter calcoaceticus IFO 12552 with stirring for, e.g. 1 to 3 days, is centrifuged so as to collect bacterial cells. Then, the collected cells are dissolved so as to prepare a lysate containing POQGDH gene. As a method for dissolving bacterial cells, for example, treatment using a lytic enzyme such as lysozyme can be employed. Furthermore, if necessary, enzymatic treatment with protease, etc. or treatment with a surface active agent such as sodium lauryl sulfate (SDS) may be carried out together. Furthermore, physical crushing method such as freezing and thawing or French press treatment may be combined.

DNA can be isolated and purified in the usual manner from the lysate obtained as mentioned above. For example, it can be carried out by appropriately combining methods such as deproteinization treatment by phenol treatment or protease treatment, ribonuclease treatment, alcohol precipitation, and the like.

In order to obtain POQGDH gene from DNA isolated and purified from microorganism, a polymerase chain reaction using a primer based on the base sequence of Acinetobacter species L.M.D 79.41 strain, which was already specified, is carried out.

Also, the amplification POQGDH gene can be cloned in an appropriate vector. As a vector to be cloned (cloning vector), a vector constructed as a recombinant vector from a phage or plasmid capable of autonomously replicating in a host microorganism is suitable. Examples of such a phage include Lambda gt10, Lambda gt11, etc. using Escherichia coli as a host organism. Similarly, examples of such a plasmid include pBR322, pUC19, pBluescript, pTet99A, pGEM-T vector, etc. using Escherichia coli as a host organism.

Cloning into a vector can be carried out by inserting POQGDH gene obtained in the above-mentioned method into a cloning site. Such an operation can be carried out by a well-known method using a restriction enzyme and DNA ligase.

The host microorganism to be used for cloning is not particularly limited as long as a recombinant vector is capable of growing stably and autonomously and expressing an exogenous gene. In general, examples of such host microorganisms include Escherichia coli W3110, Escherichia coli C600, Escherichia coli HB 101, Escherichia coli JM109, Escherichia coli DH5α, etc.

As a method for transferring a recombinant vector into a host organism, for example, in the case where, for example, the host organism is Escherichia coli, a competent cell method using a calcium treatment, electroporation, etc. can be used.

In order to select a host microorganism into which a targeted recombinant vector is introduced appropriately, a microorganism capable of expressing a marker (for example, drug resistant gene) of a vector capable of expressing GDH activity by the addition of POQ may be selected. For example, a microorganism which grows in a selective medium based on a drug resistant marker and produces POQGDH may be selected. The transformant selected in this way can produce a large amount of POQGDH stably when it is cultured in a nutritional medium.

A base sequence of a wild-type POQGDH gene obtained by the above-mentioned method is decoded by the dideoxy method described in Science, vol. 214, 1205 (1981) (see FIG. 1, SEQ ID NO: 2). Furthermore, the sequence of amino acids of POQGDH was estimated by the base sequence determined as mentioned above (see FIG. 1, SEQ ID NO: 1). Note here that sequence (SEQ ID NO: 4) and amino acid sequence (SEQ ID NO: 3), which include the signal region, are shown in FIG. 2.

The POQGDH gene collected from the recombinant vector held by the POQGDH gene can be easily modified by a genetic engineering technique. Specifically, for example, gene is modified so that amino acid residues in a certain site include substitution, deletion, insertion, addition or inversion.

<Construction of Polynucleotide Encoding Modified POQGDH>

By adding mutation to POQGDH gene obtained in this way so that certain amino acids are substituted in an expression product, genes encoding the targeted modified POQGDH can be prepared. A large number of methods for carrying out the site specific substitution of base sequence are known in this technical field (see, for example, Molecular Cloning, Third Edition, Cold Spring Harbor Laboratory Press, New York) and therefore, an appropriate method can be selected from such methods. Note here that also a random mutation is inserted into POQGDH so as to produce mutation products, and then the substrate specificities of the expression products of the mutation products (modified POQGDHs) are compared to each other. Thereby, by selecting genes having a preferable substrate specificity, gene encoding the modified POQGDH can be prepared. In a case where such a random mutation is inserted, firstly, mutation is inserted into a targeted gene region at random by using, for example, an error-prone PCR, and thus modified POQGDH gene library is constructed. A multiple substitution containing a combination of useful mutations obtained by random mutation can be obtained by a mutation scrambling method. The mutation scrambling method was reported by Norvartis Pharma in Journal of Bioscience and Industry, vol. 59, No. 3 (2001), P35-38. In this method, the modified enzyme can be evaluated simply and easily by a procedure including culturing the resultant colonies, preparing lysate of bacteria by the use of a lytic enzyme such as lysozyme, and measuring the enzymatic activity.
In order to screen the modified PQQGDH gene library efficiently, a base sequence encoding a histidine tag may be added into a PQQGDH gene in advance. Thus, despite of the change in an amount of production enzyme due to a growing state and expression efficiency, etc., enzyme activity can be evaluated by using a certain amount of enzyme. Specifically, for example, a certain amount of anti-histidine tag monoclonal antibody is adsorbed onto the surface of the substrate such as a microplate having a property of adsorbing protein, a treatment for inhibiting non-specific proteins from being adsorbed thereon is carried out, followed by reacting it with the above-mentioned lysate. Thus, a certain amount of PQQGDH contained in the above-mentioned lysate is made into a solid phase, and the enzyme activity can be evaluated. By carrying out the above-mentioned operation in a 96-well microplate, the enzyme activity of unit amount of a large number of modified PQQGDHs can be evaluated at one time efficiently.

For example, a clone holding the targeted modified gene is selected as follows. Based on the 

mock absorption at 410 nm one hour after 1-methoxy-PS and XTT are added into lysate, the activity of PQQGDH is measured. At this time, the sample whose reactivity with respect to maltose is lowered to about 80% or less as compared with the wild-type PQQGDH is firstly selected, and then a clone whose reactivity with respect to glucose is maintained at about 90% or more as compared with the wild-type PQQGDH is selected. Herein, the base sequence of the resultant clone is analyzed so as to confirm the mutation.

<Expression of Modified PQQGDH Gene>

For expression of the modified PQQGDH gene, an expression system using Escherichia coli as a host can be used. For example, firstly, the modified PQQGDH gene prepared by the above-mentioned method is inserted into a vector using Escherichia coli as a host (for example, a PUC vector, a pBluescript vector) so as to construct an expression vector. The expression vector includes a promoter sequence necessary for expressing the modified PQQGDH in the host, a replication origin, a terminator sequence, and the like.

A method for introducing (transferring) an expression vector into a host microorganism, a competent cell method using a calcium treatment, electroporation, etc. can be used. Selection of host microorganism into which an expression vector can appropriately introduced can be carried out by using the presence or absence of a drug resistant marker and the presence or absence of expression of the GDH activity when PQQ is added as indices. For example, the microorganism after introducing of an expression vector is cultured in a selection medium capable of growing only in the case where it has drug resistant marker, and then among transformants obtained to be grown, a transformant capable of producing a modified PQQGDH may be selected.

By culturing the transformant selected in the above-mentioned operation under conditions where it can be grown and proliferated and the modified PQQGDH gene inserted into an expression vector can be expressed, a large amount of modified PQQGDHs can be produced stably.

As a nutrient of a medium, nutrient that can usually be used for culturing microorganism can be used. As a carbon source, a carbon compound capable of assimilation may be used. For example, glucose, sucrose, lactose, maltose, lactose, syrup, pyruvic acid, and the like can be used. Furthermore, as a nitrogen source, a usable nitrogen compound can be used. For example, peptone, meat extract, yeast extract, casein hydrolysate, soybean cake alkaline extract, and the like, can be used. Besides, salts such as phosphate, carbonate, sulfate, magnesium, calcium, potassium, iron, manganese, zinc, etc., certain amino acids, certain vitamin, and the like, can be used if necessary.

Transformant is cultured under temperature conditions where the transformant can be grown and modified PQQGDH can be produced. For example, the culture temperature can be set at 20°C to 30°C. The cultivating period can be set in consideration of the growing property of the transformant to be cultured, the production property of the modified PQQGDH, or the necessary production amount of the modified PQQGDH and the like. For example, at the time when the yield of the modified PQQGDH reaches maximum, the culture is completed. The cultivating period may be about 12 to 72 hours. pH of the medium is adjusted to the range in which the transformant can grow and modified PQQGDH can be produced. Preferred pH is about 6.0 to 9.0.

The culture medium containing cell bodies producing modified PQQGDH can be used as they are or used after they are subjected to treatment such as concentration treatment or treatment for removing impurities, etc. However, in general, the modified PQQGDH is collected from the culture medium or cell bodies. When the produced modified PQQGDH is a secretion type protein, it can be collected from the culture medium; and the produced modified PQQGDH is other than the secretion type protein, it can be collected from the cell bodies. In case that the modified PQQGDH is collected from the culture medium, for example, culture supernatant is filtrated, subjected to centrifugal treatment so as to remove impurities, followed by carrying out vacuum concentration, membrane concentration, salting out using ammonium sulfate or sodium sulfate, fractional precipitation method by using methanol, ethanol, acetone, or the like, dialysis, heat treatment, iso-electric treatment, various kinds of chromatography such as gel filtration or adsorption chromatography, ion exchange chromatography, affinity chromatography, etc. (for example, gel filtration by using Sephadex gel (Pharmacia Biotech), etc., DEAE sepharose CL-6B (Pharmacia Biotech), octyl sepharose CL-6B (Pharmacia Biotech), CM sepharose CL-6B (Pharmacia Biotech)), and the combination thereof so as to isolate and purify modified PQQGDH product. Thus purified product of the modified PQQGDH can be obtained. On the other hand, when the modified PQQGDH is collected from cell body, by carrying out the filtration and centrifugal treatment, etc. of culture medium, cell bodies are collected, and then the cell bodies are crushed by a mechanical method such as a pressure treatment, an ultrasonic treatment, etc. or by an enzymatic method using lysozyme etc. Thereafter, similar to the above, isolation and purification is carried out so as to obtain a purified product of the modified PQQGDH. Note here that if necessary, by adding a chelating agent such as EDTA and a surface active agent, PQQGDH may be solubilized and PQQGDH can be isolated and collected as an aqueous solution.

It is preferable that the purified enzyme is purified to such an extent that it shows a single band in an electrophoresis (SDS-PAGE).

The purified enzyme obtained as mentioned above can be distributed in a state of particles by, for example, freeze-drying, vacuum drying or spray drying, and the like. At this time, the purified enzyme may be dissolved in a phosphate buffer solution, a TRIS hydrochloric acid buffer solution, or Good’s buffer solution in advance. The suitable buffer solution to be used herein is a Good’s buffer, and above all, PIPES, MES or MOPS buffer solution is particularly preferable.

The modified PQQGDH of the present invention catalyzes a reaction for producing 8-gluconolactone by oxidizing glucose using PQQ as a coenzyme. Such an enzyme activity can be determined by measuring an amount of PQQ reduced in accordance with oxidation of glucose by PQQGDH by a color reaction with a redox reagent. As the
color reagent, for example, PMS-DCIP, 1-methoxy-PMS-XTT, potassium ferricyanide, and the like can be used.

In the present invention, in principle, the PQQGDH activity can be measured by using the following reagent under the following conditions.

<Reagent>
10 mM MOPS (pH 7.0)
1 mM 1-methoxy PMS (phenazine methosulfate)
0.25 mM XTT
5 mM glucose

<Measurement Conditions>
140 μl of the above-mentioned reagent mixing solution is preheated at 25°C. for about 5 minutes, followed by adding 0.1 ml of enzyme solution to calmly admix thereof. Thereafter, the admixture is subject to recordation for 15 minutes by a spectrophotometer controlled at 25°C. with water as a control and from the linear part, change in absorbance at 410 nm for 1 minute is measured. In a blind test, instead of enzyme solution, distilled water is added in a test mixture, and change in absorbance of light with 410 nm for 1 minute is similarly measured. In the above-mentioned conditions, the amount of enzyme of 1/2 μmol of formazan produced per minute is made to be 1 unit (U).

The selectivity with respect to glucose of the modified PQQGDH of the present invention can be evaluated as follows. That is to say, enzyme activities when the substrate are various kinds of sugars such as lactose, maltose, galactose, sucrose and xylose are examined in the similar way to the above. The resultant enzyme activities are expressed as relative values relate to the activity when the substrate is glucose. Thus, the relative activities can be evaluated.

By using the modified PQQGDH of the present invention, it is possible to construct a glucose measurement kit. That is to say, another aspect of the present invention provides a glucose measurement kit containing the modified PQQGDH. In addition to the modified PQQGDH, such a kit can contain a solution such as a buffer solution and a glucose solution as a standard, etc. which are necessary for measurement.

The following examples further illustrate the present invention. These examples are not intended to limit the scope of the present invention.

EXAMPLE 1
Isolation of Chromosomal DNA

Chromosomal DNA of Acinetobacter calcoaceticus IFO 12552 strain was isolated by the following method. This strain was cultured in 10 mL of LB medium with shaking at 30°C. over night, and then centrifuged (at 15000 rpm for 10 minutes) so as to collect bacteria. From the collected bacteria, chromosomal DNA was extracted and purified by using a Dneasy tissue kit (QUIGEN K.K.) and dissolved in a TE buffer.

EXAMPLE 2
Preparation of DNA Fragment Containing Gene Encoding PQQGDH, and Construction of Recombinant Vector Containing DNA Fragment

The chromosomal DNA obtained in Example 1 was used as a template to amplify a region of DNA including the targeted PQQGDH gene by polymerase chain reaction (PCR) using the following primers. Note here that the primers to be used herein were designed based on a base sequence of soluble PQQGDH (A-M Cleton-Jansen et al., Gen. Genet., 217, 430 (1989)) derived from Acinetobacter species L.M.D 79.41 strain.

Forward Primer:
5'-ACAAATCATATAGAAGACTG-3' (SEQ ID NO: 5)
Reverse Primer:
5'-TTACTTAGCCTTATAGGTTAAGTATCAGAGATCCGAGG-3' (SEQ ID NO: 6)

The PCR was carried out in a solution having the composition shown in Table 1 under conditions of carrying out a reaction at 94°C. for 2 minutes, then 30 cycles of reactions at 94°C. for 30 seconds, at 55°C. for 30 seconds and at 72°C. for 2 minutes, and finally a reaction at 72°C. for 10 minutes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAKARA LA-taq;</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>10-fold buffer;</td>
<td>5 μL</td>
</tr>
<tr>
<td>25 mM MgCl₂;</td>
<td>5 μL</td>
</tr>
<tr>
<td>dNTP mix (2.5 mM);</td>
<td>8 μL</td>
</tr>
<tr>
<td>Forward primer (10 pmol/μl);</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse primer (10 pmol/μl);</td>
<td>1 μL</td>
</tr>
<tr>
<td>template adjusted to 50 μL by H₂O</td>
<td></td>
</tr>
</tbody>
</table>

The resultant amplification gene fragment was ligated to a pGEM-T Easy vector (QUIGEN K.K.) and Escherichia coli JM109 strain was transformed in this plasmid. Note here that the resultant plasmid was defined as pTGM-GDH.

EXAMPLE 3
Determination of Sequence List of Soluble PQQGDH of Acinetobacter calcoaceticus IFO 12552 Strain

The sequence of the gene fragment inserted into the plasmid pTGM-GDH obtained in Example 2 was determined by the use of BigDye Terminator Sequencing Kit (Applied Biosystems Japan Ltd.). The determined base sequence and amino acid sequence are shown in SEQ ID NO: 2 and SEQ ID NO: 1, respectively. Note here that base sequence and amino acid sequence including the signal regions are shown in SEQ ID NO: 4 and SEQ ID NO: 3, respectively. The molecular weight of protein calculated from the amino acid sequence was about 50,000 which was substantially identical with the molecular weight of soluble PQQGDH of Acinetobacter calcoaceticus.

EXAMPLE 4
Construction of Expression Vector Containing PQQGDH Gene

The plasmid pTGM-GDH obtained in Example 2 was used as a template and polymerase chain reaction (PCR) using the following primers was carried out.

Forward primer: 5'-GGGCGGCGTAGACTATGAATACATTTGCTA AAATATTTTATATG-3' (SEQ ID NO: 7)
Reverse primer: 5'-GGGCGGCGCTGACAGTATTACACTTCGATGCTGCT AGTCAATATGAGATTACATCTGGG-3' (SEQ ID NO: 8)

The PCR was carried out in a solution having the composition shown in Table 2 under conditions of carrying out a reaction at 94°C. for 2 minutes, then 30 cycles of reactions at 94°C. for 30 seconds, at 55°C. for 30 seconds and at 72°C. for 2 minutes, and finally a reaction at 72°C. for 10 minutes.
TABLE 2

<table>
<thead>
<tr>
<th>Sigma KlenTag:</th>
<th>0.5 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-fold buffer:</td>
<td>5 µL</td>
</tr>
<tr>
<td>dNTP mix (10 mM):</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Forward primer (10 pmol/µL):</td>
<td>2 µL</td>
</tr>
<tr>
<td>Reverse primer (10 pmol/µL):</td>
<td>2 µL</td>
</tr>
<tr>
<td>template adjusted to 50 µL by H₂O</td>
<td></td>
</tr>
</tbody>
</table>

The resultant amplification gene was cut by restriction enzymes EcoRI and PstI. The cut gene fragment was ligated to an expression vector. Escherichia coli JM109 strain was transformed with this plasmid. Note here that the resultant expression plasmid was defined as pUK-GDHB(S) (see FIG. 3). When the transformant was cultured in 100 mL of LB medium containing 100 µg/mL of ampicillin and 6 µM POQ at 30°C over night, production of PQQGDH was observed. Note here that the molecular weight of this PQQGDH calculated from the amino acid sequence was about 50,000 which was substantially identical to the molecular weight of Acinetobacter calcoaceticus IFO 12552.

EXAMPLE 5

Production of Acinetobacter calcoaceticus PQQGDH Gene Product

50 mL each of LB medium was dispensed in a 500 mL vol flask and subjected to autoclaving at 121°C for 20 minutes. After cooling down, to the medium, 100 mg/mL ampicillin solution and isopropyl galactopyranoside (50 µL), which were separately filtrated aseptically, were added and a colony having a PQQGDH expression vector pUK-GDHB(S) was inoculated. Then, the medium was cultured with shaking at 30°C for 16 hours. When the culture was completed, the PQQGDH activity was about 1 U/mL.

After culturing, the medium was centrifuged so as to collect bacteria. The collected bacteria were suspended in 10 mM Tris-HCL (pH 7.5). Then, the bacteria suspension was ultrasonic disintegrated and then centrifuged again so as to obtain a supernatant as a crude enzyme solution. This crude enzyme solution was treated with a cationic coagulation agent and concentrated by desalting, followed by separating and purifying by an DEAE sepharose (Pharmacia Biotech), CM sepharose (Pharmacia Biotech) column chromatography. Thus, purified enzyme was obtained.

The PQQGDHs obtained by the above-mentioned method showed a substantially single band in electrophoresis. The specific activity at this time was about 1600 U/mg. Hereinafter, properties of the resultant PQQGDH are described.

Action: D-glucose+artificial electron acceptor →d-glucuronolactone+reduced electron acceptor

Thermal stability: 50°C (pH: 7.0, treatment for 10 minutes)

pH stability: pH 6 to 9 (30°C, treatment for one hour)

Optimum temperature: about 50°C

Optimum pH: 7.0

Molecular weight: 50,000

EXAMPLE 6

Addition of C-Terminal Histidine Tag of PQQGDH

Firstly, a histidine tag was introduced in the downstream of PQQGDH gene. Then, a PCR was carried out using a primer for adding a stop codon right behind it. A PCR using the following primers was carried out using pUK-GDHB(S) obtained in Example 4 as a template.

Forward primer: 5'-TCACAATGGTTCCTTCCTGCCATATCATC-3' (SEQ ID NO: 9)

Reverse primer: 5'-ATGGTGATGGTGATGGTGCTTAGCCCTATAAGGTGAA CTATAAAGAA-3' (SEQ ID NO: 10; underline is given to the sequence of 6x histidine tag)

The PCR was carried out under the same conditions as in Example 4.

Then, the PCR using the following primers was carried out in order to introduce a translation stop codon in the downstream of a histidine tag by using an amplified gene as a template.

Forward primer: 5'-TCACAATGGTTCCTTCCTGCCATATCATC-3' (SEQ ID NO: 9)

Reverse primer: 5'-GGCGCGTACTATGTTGTCGTTGCTTAGCCCTATAAGGTGAA CTATAAAGAA-3' (SEQ ID NO: 11)

The PCR was carried out by using the same solution composition and under the same conditions as in Example 4.

The amplified gene obtained by adding a histidine tag and a stop codon in the downstream of PQQGDH was cut with EcoRI and PstI. The cut gene fragment was ligated to an expression vector so as to construct a plasmid. Escherichia coli JM109 strain was transformed with this plasmid. Note here that the resultant expression plasmid was defined as pUK-GDHB(S)-His. When the transformant was cultured in 100 mL of LB medium containing 100 µg/mL of ampicillin and 6 µM POQ at 30°C over night, the production of PQQGDH was observed. Note here that the molecular weight of this PQQGDH calculated from the amino acid sequence was about 50,000 which was substantially identical to the molecular weight of Acinetobacter calcoaceticus IFO 12552. Furthermore, when general properties were compared with the wild-type PQQGDH obtained in Example 5 as a control, they were found to be equal to each other.

EXAMPLE 7

Construction of Mutant PQQGDH Gene Library

The plasmid PUK-GDHB(S)-His obtained in Example 6 was used as a template to introduce random mutations by error-prone PCR using the following primers.

Forward primer: 5'-TCACAATGGTTCCTTCCTGCCATATCATC-3' (SEQ ID NO: 9)

Reverse primer: 5'-GGCGCGTACTATGTTGTCGTTGCTTAGCCCTATAAGGTGAA CTATAAAGAA-3' (SEQ ID NO: 12)

The conditions for introducing mutations are mentioned below. The PCR was carried out in a solution having the composition shown in Table 3 under conditions of carrying out a reaction at 94°C for 5 minutes, then 25 cycles of reactions at 94°C for one minute, 50°C for one minute and 70°C for 2 minutes, and finally a reaction at 72°C for 7 minutes.

TABLE 3

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris-HCL (pH 8.3)</td>
<td>5 µL</td>
<td></td>
</tr>
<tr>
<td>500 mM KCl</td>
<td>5 µL</td>
<td></td>
</tr>
<tr>
<td>1 mg/mL BSA</td>
<td>2.5 µL</td>
<td></td>
</tr>
<tr>
<td>(15–100 mM) MgCl₂</td>
<td>5 µL</td>
<td></td>
</tr>
<tr>
<td>1 mM MnCl₂</td>
<td>1.25 µL, to 3.75 µL</td>
<td></td>
</tr>
<tr>
<td>10 mM ATP</td>
<td>0.3 µL, to 1.55 µL</td>
<td></td>
</tr>
<tr>
<td>10 mM TTP</td>
<td>0.5 µL, to 5.0 µL</td>
<td></td>
</tr>
<tr>
<td>10 mM GTP</td>
<td>1.0 µL, to 5.0 µL</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 3-continued

| 10 mM CTP:  | 0.5 μL to 5.0 μL |
| Forward primer (20 pmol/μL): | 2.5 μL |
| Reverse primer (20 pmol/μL): | 2.5 μL |
| Dimethylsulfoxide: | 0.2 mg/L template: 0 to 7.5 μL |
| Taq Polymerase: | 0.2 mg/L template: 5 μL |
| 10 mM CTP: | 0.5 μL to 5.0 μL |
| Forward primer (20 pmol/μL): | 2.5 μL |
| Reverse primer (20 pmol/μL): | 2.5 μL |
| Dimethylsulfoxide: | 0.2 mg/L template: 0 to 7.5 μL |
| Taq Polymerase: | 0.2 mg/L template: 5 μL |

The amplified gene fragment into which the mutation was introduced was cut with EcoRI and PstI so as to be substituted by the wild-type gene of pUK-GDHB(S). Escherichia coli JM109 was transformed with the thus obtained plasmid so as to construct a mutant gene library.

The resultant colony was inoculated into 600 μL of Circle Grow Medium (Stratagene) in a 96-well culturing microplate having a deep well. The Circle Grow Medium contained 10 μg/mL ampicillin, 5 μM of PQG and 1 mM calcium chloride. The medium was cultured with shaking at 200 rpm at 37°C overnight. Thereafter, bacteria were collected through a centrifuge (at 3000 rpm, 4°C, 30 minutes) and suspended in 600 μL of three-times diluted lysate (0 mM TRIS buffer solution, pH 8.0, 2 mM EDTA, 1 mg/mL lysozyme, 15 μM PQG) again. Then, the suspension was subjected to shaking at 37°C for one hour and 30 μL of 20 mM calcium chloride solution was added therein. The lysate was transferred to a filter plate (Corning) and centrifuged (at 2000 rpm, 4°C for 5 minutes). The resultant supernatant was used for screening mentioned below.

A 96-well microplate to be used for screening was prepared in the following method in advance. That is to say, firstly, 100 μL of 15 ng/mL Protein A/G solution was added in a 96-well microplate (Coaster Co., Ltd.) and allowed it stand at 37°C for about 2 hours, then washed with a PBS solution three times, added by 150 μL of a blocking solution and allowed it stand at 37°C for about 2 hours. Thus, a microplate for screening was prepared. Into a 96-well microplate containing 80 μL of 0.1 ng/mL mouse IgG1 anti 5x histidine tag monoclonal antibody was prepared in advance, the above-mentioned supernatant was added and admixed, followed by adding into a screening microplate from which 100 μL of blocking solution was washed to be removed with a PBS solution and allowed it stand 25°C for 1 hour. Thereafter, the microplate was washed with PBS three times and added by 140 μL of PQG assay solution (mixing solution containing 3.5 mL of 4 mM 1-methoxy PMS, 3.5 mL of 1 mM XTT solution, 3.5 mL of 20 mM maltose or glucose solution, 3.5 mL of 40 mM MOPS—NaOH, pH 7.0) and the absorbance at 410 nm was measured at room temperature for 15 minutes.

In the first stage of the screening, a random one-base substitution library was screened with maltose as a substrate so as to identify a clone for expressing a mutant whose enzyme activity was lowered by about 80% or less of that of the wild-type enzyme. The identified mutant strain was screened so as to obtain a clone expressing a mutant whose reactivity with respect to maltose is lower than that with respect to glucose. When the base sequence of these mutant PQG genes was determined, it was clarified that glycine at position 75 was mutated to arginine, glycine at position 295 was mutated to glutamic acid, methionine at position 342 was mutated to valine, alanine at position 351 was mutated to threonine; glutamine at position 168 was mutated to histidine; leucine at position 169 was mutated to glutamine; and tryptophan at position 347 was mutated to arginine. As shown Table 4 below, the modified PQG DH had lowered specificity to maltose as compared with that of the wild-type. The activity of the wild-type PQG DH using glucose as a substrate was made 100, and with respect to this, the reactivity with respect to maltose was shown. Note here that as a control, a wild-type PQG DH added by a histidine tag obtained in Example 6 was used.

In Table 4, numbers in the column of amino acid substitution represent amino acid number and letters written at the left of the numbers are kinds of amino acids in a wild-type and letters at the right of the numbers are kinds of amino acids after being mutated. Therefore, for example, Gly75 Arg means a modified product in which glycine that is an amino acid at position 75 was mutated to arginine.

### TABLE 4

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>glucose</th>
<th>Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>Gly75Arg</td>
<td>100</td>
<td>72</td>
</tr>
<tr>
<td>Glu168His</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>Lue169Gln</td>
<td>100</td>
<td>63</td>
</tr>
<tr>
<td>Gly295Cina</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>Met342Val</td>
<td>100</td>
<td>69</td>
</tr>
<tr>
<td>Trp347Tla</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>Ala351Thr</td>
<td>100</td>
<td>59</td>
</tr>
</tbody>
</table>

### EXAMPLE 8

Introduction of Site-Specific Mutation into Wild-Type PQG DH and Consideration of Substrate Specificity of Modified PQG DH

A site-specific mutation was introduced into a structural gene of PQG DH derived from Acinetobacter calcoaceticus shown by SEQ ID NO: 4 by using a QuickChange Site-directed Mutagenesis kit (Stratagene). The introduction of mutation was carried out under conditions where a base sequence encoding the amino acids at sites into which the mutation was introduced in Example 7, that is, glycine at position 75, glycine at position 295, methionine at position 342, alanine at position 351, glutamine at position 168, leucine at position 169 and tryptophan at position 347 are substituted so as to encode 20 kinds of amino acids at random. The sequences of primers for substituting the sites by 20 kinds of amino acids are shown in Table 5. Note here that in Table 5, F and R which are given to the end sequence names represent forward and reverse, respectively.

### TABLE 5

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>sequence</th>
<th>(35 mer, SEQ ID NO: 13)</th>
<th>(35 mer, SEQ ID NO: 14)</th>
<th>(38 mer, SEQ ID NO: 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDDH/G75-F</td>
<td>5'-GTAAATGATGCTGATNNNCAAACGTTTATTGOG-3'</td>
<td>5'-CCCAAAAAACCGTTTTTGGNNNTACGACATCATTTAC-3'</td>
<td>5'-CAAATTAAAGATTTANNNCAAAAATGTTTAAAAGTGGC-3'</td>
<td></td>
</tr>
</tbody>
</table>
The modified PQQGDHs have lowered reactivity with respect to both maltose and galactose. Furthermore, at the same time, the modified PQQGDH having lowered reactivity with respect to lactose and galactose was obtained. Note here that in Table 6, numbers in the column of amino acid substitution represent amino acid number and letters written at the left of the numbers are kinds of amino acids in a wild-type and letters at the right of the numbers are kinds of amino acids after being mutated. Therefore, for example, Gly75Arg means a modified product in which glycine that is an amino acid at position 75 was mutated to arginine.

**Table 6**

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>Glucose</th>
<th>Maltose</th>
<th>Lactose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>90</td>
<td>75</td>
<td>34</td>
</tr>
<tr>
<td>Gly75Trp</td>
<td>100</td>
<td>78</td>
<td>88</td>
<td>42</td>
</tr>
<tr>
<td>Gln168Ser</td>
<td>100</td>
<td>35</td>
<td>64</td>
<td>16</td>
</tr>
<tr>
<td>Gln168Gly</td>
<td>100</td>
<td>24</td>
<td>63</td>
<td>12</td>
</tr>
<tr>
<td>Gln168Trp</td>
<td>100</td>
<td>74</td>
<td>69</td>
<td>23</td>
</tr>
<tr>
<td>Leu169Phe</td>
<td>100</td>
<td>48</td>
<td>78</td>
<td>33</td>
</tr>
<tr>
<td>Gly295Cys</td>
<td>100</td>
<td>80</td>
<td>74</td>
<td>36</td>
</tr>
<tr>
<td>Gly295Asp</td>
<td>100</td>
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<td>77</td>
<td>36</td>
</tr>
<tr>
<td>Gly295Glu</td>
<td>100</td>
<td>75</td>
<td>72</td>
<td>37</td>
</tr>
<tr>
<td>Gly295Phe</td>
<td>100</td>
<td>75</td>
<td>70</td>
<td>34</td>
</tr>
<tr>
<td>Gly295Val</td>
<td>100</td>
<td>63</td>
<td>65</td>
<td>37</td>
</tr>
<tr>
<td>Gly295Tyr</td>
<td>100</td>
<td>32</td>
<td>81</td>
<td>28</td>
</tr>
<tr>
<td>Met342Pro</td>
<td>100</td>
<td>78</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>Trp347His</td>
<td>100</td>
<td>86</td>
<td>90</td>
<td>57</td>
</tr>
<tr>
<td>Ala351Thr</td>
<td>100</td>
<td>60</td>
<td>86</td>
<td>21</td>
</tr>
</tbody>
</table>

**Example 9**

Introduction of Multiple Mutation Using Mutation Scrambling Method and Consideration of Substrate Specificity of Multiple Substitution

A library of multiple-substituted products having any possible combinations of amino acid substitutions effective in improving the substrate specificity which was apparent from Example 8 was prepared by a mutation scrambling method, thereby attempting to obtain a modified product having higher substrate specificity as compared with the case where only one amino acid substitution is contained. The multiple substitution library was prepared by a biased mutation scrambling method which had already been reported was employed (see the 39th Annual Meeting of Biophysical Society of Japan held at Oct. 6, 2001; Subject No: 1PO4, Novartis Pharma K. K.; Biopolymers, Vol. 64 (2002), P. 95–105). The biased mutation scrambling method is a modified method of the mutation scrambling method and has a feature capable of freely setting the introduction rate.
of amino acid substitutions to be combined. In this Example, the rate was set under conditions where G75, G295, M342 and A351 which were located in the vicinity of the substrate binding sites by estimation from a structure model and particularly had high contribution to the substrate specificity included mutations at high rate of 80% and Q106, L169 and W347 which were thought to have structural features include mutations at the rate of 50%. Specifically, PQQGDH gene was reconstructed by a mutation scrambling method so that the full length of the PQQGDH was divided into 5 fragments shown in FIG. 4 including 1 to 3 amino acid substitutions which were overlapped at the terminal portions and identified in Example 8 were introduced at the above-mentioned rate.

In the mutation scrambling method, firstly, a PCR was carried out using a wild-type gene as a template so as to obtain a partial amplified product (see FIG. 4). The compositions of the reaction solution used in the PCR are shown in Table 7. As the primer, a primer sandwiching G75 and including a sequence corresponding to Q106 and L169 was used.

### Table 7

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer for AccuTaq (SIGMA CORPORATION)</td>
<td>2</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>1</td>
</tr>
<tr>
<td>Forward primer (10 pmol/μL)</td>
<td>1.2</td>
</tr>
<tr>
<td>Reverse primer (10 pmol/μL)</td>
<td>1.2</td>
</tr>
<tr>
<td>Template (10 ng/μL)</td>
<td>0.5</td>
</tr>
<tr>
<td>dNTP</td>
<td>13.9</td>
</tr>
<tr>
<td>AccuTaq (5 units/μL)</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

Forward primer: 5'-TCACATGTCTTTTCTGCTGGTATC-3' (SEQ ID NO: 9)
S-primer (G)-10-QL: 5'-AAgAAATAAATAAGCCCaTGgT-TgCggTCCCTGATC-3' (SEQ ID NO: 27)

The reaction was carried out under conditions of 98° C. for 30 seconds, 30 cycles of 94° C. for 5 seconds, 60° C. for 20 seconds, and 68° C. for 90 seconds, and finally 68° C. for 10 minutes. Then, under the same condition, a PCR was carried out by using a modified gene having a mutation at G75 as a template so as to obtain a partial amplified product of the same region as mentioned above. Herein, as shown in Table 6, the number of combinations of the amino acid substitutions at sites G75, Q168 and L169 is 16. The partial amplified products corresponding to all these combinations were prepared by carrying out a reaction using 8 kinds of primers (S-primer(G)-10-QL to S-primer(G)-10-YF) shown in FIG. 4 and using two kinds of genes of wild-type gene and modified type gene as templates. The resultant partial amplified products were mixed at the mixing ratio shown in FIG. 5 so as to obtain a fragment 1 (see fragment 1 in FIG. 4). Fragments 2 to 5 (see fragment 2, fragment 3, fragment 4 and fragment 5 in FIG. 4) were prepared by mixing gene amplified products obtained by using a primer shown in FIG. 4 in the same manner for fragment 1 at the ratio shown in FIG. 5 respectively.

Then, equal amount of each fragment was mixed and a PCR was carried out in a solution having the composition shown in Table 8. The reaction was carried out under conditions of 98° C. for 30 seconds, and 5 cycles of 94° C. for 5 seconds, 60° C. for 20 seconds, and 68° C. for 2 minutes. After the PCR was completed, 2.4 μL each of the above-mentioned primer (10 pmol/μL) (SEQ ID NOs: 7 and 8) was added and a PCR was carried out again. At this time, the reaction was carried out under conditions of 94° C. for 5 seconds, 25 cycles of 60° C. for 20 seconds and 68° C. for 2 minutes, and finally 72° C. for 10 minutes.

### Table 8

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X buffer for AccuTaq (SIGMA CORPORATION)</td>
<td>2</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>1</td>
</tr>
<tr>
<td>dNTP</td>
<td>(14.4–X) μL</td>
</tr>
<tr>
<td>AccuTaq (5 units/μL)</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>17.6 μL</td>
</tr>
</tbody>
</table>

The library of the modified gene obtained by the above-mentioned operation was cut by EcoRI and PstI and substituted by a wild-type gene of pUK-GDHB(S), and then transformation of Escherichia coli was performed. Colony of the transformants were cultured in the method shown in Example 8, then screening was carried out with the PQQGDH activity and the substrate specificity as indices. For clones showing the improvement in the substrate specificity, the base sequence of the modified PQQGDH held by the clone was determined. As a result, as shown in Table 6, it was found that a plurality of modified PQQGDHs having different kinds of combinations of amino acids to be substituted. Note here that in Table 6, a mark such as G75 (GGA) means the number specifies the position of an amino acid and one letter of alphabet at the left of the number represents an amino acid at the position. Furthermore, alphabet in parenthesis means the base sequence encoding the amino acid. On the other hand, a mark such as Phe (TTT) shows substituted product in which the mark (for example, L194 (CTG)) that is located at the top part of the column to which the Phe (TTT) belongs is substituted by an amino acid specified by three letters of alphabet. Furthermore, letters in parenthesis means the base sequence encoding the amino acid.

After each transformant having these modified PQQGDH was purified by the same method as in Example 7, the reactivity with respect to substrate was measured. The measurement results are shown in the right column of FIG. 6. As is apparent from this Table, in any of the obtained modified PQQGDHs, the reactivity with respect to maltose was significantly lowered as compared with that of the wild type PQQGDH. Furthermore, as to the reactivity with respect to lactose, the reactivity was found to be lowered similarly. Furthermore, the reactivity with respect to galactose was greatly lowered in any of the modified PQQGDHs, and in some of the modified PQQGDH, the reactivity was extremely lowered. In this way, we successfully obtained modified PQQGDH having the significantly lowered reactivity with respect to maltose, lactose and galactose, that is, having an extremely excellent substrate specificity. As a result, by combining the mutations at the positions of the amino acid shown in Table of FIG. 6, it was found that the modified PQQGDH having more excellent substrate specificity could be prepared as compared with the case where the mutation is a single.

Herein, the amino acid sequence of No. 1 modified product is shown in SEQ ID NO: 28; the amino acid sequence of No. 2 modified product is shown in SEQ ID NO: 29; the amino acid sequence of No. 3 modified product is shown in SEQ ID NO: 30; the amino acid sequence of No. 4 modified product is shown in SEQ ID NO: 31; the amino acid sequence of No. 5 modified product is shown in SEQ ID NO: 32; the amino acid sequence of No. 6 modified
product was shown in SEQ ID NO: 33; and the amino acid sequence of No. 7 modified product was shown in SEQ ID NO: 34, respectively.

In particular, in the No. 1 modified product, the reactivity with respect to maltose is lowered to about \( \frac{7}{8} \) of the wild-type, and similarly the reactivity with respect to lactose is lowered to about \( \frac{5}{8} \), and the reactivity with respect to galactose is lowered about \( \frac{3}{4} \), respectively. Furthermore, also in the No. 6 modified product, the reactivity with respect to maltose is lowered to about \( \frac{1}{2} \) of the wild-type, and at the same time, the reactivity with respect to lactose is also lowered, and the reactivity with respect to galactose is lowered about \( \frac{1}{4} \), respectively. Similarly, also in the No. 7 modified product, the reactivity with respect to maltose is lowered to about \( \frac{1}{3} \) of the wild-type, and at the same time, the reactivity with respect to lactose is also lowered to about \( \frac{1}{5} \). Substantial reactivity is not shown with respect to galactose. From the comparison of the amino acid substitution sites of the No. 1, No. 6 and No. 7 modified products, it is suggested that the substitution of the amino acid at position 342 and the amino acid at position 351 are particularly important for improvement of the substrate specificity. In particular, it is expected that when amino acid are substituted in both positions, high substrate specificity can be obtained. Furthermore, also the substitution of the amino acid at position 295 is also important, and further, the substitution of the amino acid at position 169 is greatly involved in the improvement of the substrate specificity.

The present invention is not limited only to the description of the above embodiments. A variety of modifications which are within the scopes of the following claims and which are achieved easily by a person skilled in the art are included in the present invention.

---

**SEQUENCE LISTING**

- **<160> NUMBER OF SEQ ID NOS:** 34
- **<210> SEQ ID NO 1
  **<211> LENGTH:** 455
  **<212> TYPE:** PRT
  **<213> ORGANISM:** Acinetobacter calcoaceticus
- **<400> SEQUENCE:** 1

```
Asp Val Pro Leu Thr Pro Ser Gln Phe Ala Lys Ala Lys Thr Glu Ser
1      5
Phe Asp Lys Val Leu Ser Ser Leu Asn Leu Asn Lys Pro His Ala Leu
20     25
Leu Trp Gly Pro Asp Asn Gln Ile Trp Leu Thr Glu Arg Ala Thr Gly
35     40
Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe
50     55
Gln Val Pro Glu Ile Val Asn Asp Ala Asp Gly Gln Asn Gly Leu Leu
65     70
Gly Phe Ala Phe His Pro Asp Phe Lys Asn Pro Tyr Ile Tyr Val
85     90
Ser Gly Thr Phe Lys Pro Lys Ser Ser Thr Asp Lys Glu Leu Pro Asn
100    105
Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ala Thr Asp Thr Leu
115    120
Glu Lys Pro Val Asp Leu Leu Ala Gly Leu Pro Ser Ser Lys Asp His
130    135
Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Thr Thr
145    150
Ile Gly Asp Gln Gly Arg Asn Gln Ala Tyr Leu Phe Leu Pro Asn
165    170
Gln Ala Gln His Thr Pro Thr Gln Glu Leu Ser Gly Lys Asp Tyr
180    185
His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile
195    200
Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Ile Ser His Ile Tyr Thr
210    215
Leu Gly His Arg Asn Pro Glu Gly Leu Ala Phe Thr Pro Asn Gly Lys
225    230
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<th>Position</th>
<th>Sequence</th>
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<td>285</td>
<td></td>
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<td>290</td>
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<223> OTHER INFORMATION: Description of Artificial Sequence : sense primer

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SEQ ID NO 10
LENGTH: 46
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: antisense primer

SEQUENCE: 10
atggtgatgg tgaatggtgcc tagcctata ggtgaacct ataaga

SEQ ID NO 11
LENGTH: 42
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: antisense primer

SEQUENCE: 11
gcggtgcgct gcgcgcattata atggtgatgg tgcctagcct ta

SEQ ID NO 12
LENGTH: 23
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: sense primer G6H8/G75-F

SEQUENCE: 12
ggcggtact atggtgtgcct tga

SEQ ID NO 13
LENGTH: 35
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: antisense primer G6H8/G75-R

SEQUENCE: 13
gttaatagtg ctagttnnnnc aaacagttta ttgyg

SEQ ID NO 14
LENGTH: 35
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: n stands for any base

SEQUENCE: 14
cocaaaaaac cgttttgmn atcgacota tttac

SEQ ID NO 15
LENGTH: 30
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: sense primer GDEB/G295-F
FEATURE:
NAME/KEY: misc_feature
LOCATION: (16)..(18)
OTHER INFORMATION: n stands for any base

SEQUENCE: 15
caatnccg atttannnc aastggtta aasgtgc

SEQ ID NO 16
LENGTH: 38
TYPE: DNA
ORGANISM: Artificial Sequence
OTHER INFORMATION: Description of Artificial Sequence: antisense primer GDEB/G295-R
FEATURE:
NAME/KEY: misc_feature
LOCATION: (21)..(23)
OTHER INFORMATION: n stands for any base

SEQUENCE: 16
gccttcttc aacacttttg nntntaatct ttatattg

SEQ ID NO 17
LENGTH: 39
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: sense primer GDEB/M342-F
FEATURE:
NAME/KEY: misc_feature
LOCATION: (16)..(18)
OTHER INFORMATION: n stands for any base

SEQUENCE: 17
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SEQ ID NO 18
LENGTH: 33
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: antisense primer GDEB/M342-R
FEATURE:
NAME/KEY: misc_feature
LOCATION: (16)..(18)
OTHER INFORMATION: n stands for any base

SEQUENCE: 18
caacacacagt tagtttnnac cccacacagt tgg

SEQ ID NO 19
LENGTH: 33
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: sense primer GDEB/AJ51-F
FEATURE:
NAME/KEY: misc_feature
LOCATION: (11)..(13)
OTHER INFORMATION: n stands for any base

SEQUENCE: 19
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LOCATION: (18), (20)
OTHER INFORMATION: n stands for any base

SEQUENCE: 24

gtaagaata aataagonn ctggtacgc ccc

SEQ ID NO 25
LENGTH: 37
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: sense primer GDRB/W347-F
NAME/KEY: misc_feature
LOCATION: (20), (22)
OTHER INFORMATION: n stands for any base

SEQUENCE: 25

ggtatgacc tacattttgcn nnccacaggt tgcgocg

SEQ ID NO 26
LENGTH: 37
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: antisense primer GDRB/W347-R
NAME/KEY: misc_feature
LOCATION: (16), (18)
OTHER INFORMATION: n stands for any base

SEQUENCE: 26
cgagacgcc agttgmnncg aasttaggt cstatcc

SEQ ID NO 27
LENGTH: 35
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence: antisense primer

SEQUENCE: 27
agastaeast aacccagttg gttacgcccc tgcgcc

SEQ ID NO 28
LENGTH: 455
TYPE: PRT
ORGANISM: Acinetobacter calcoaceticus
FEATURE:
NAME/KEY: MUTAGEN
LOCATION: (169), (169)
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NAME/KEY: MUTAGEN
LOCATION: (295), (295)
OTHER INFORMATION:
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NAME/KEY: MUTAGEN
LOCATION: (342), (342)
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LOCATION: (351), (351)
OTHER INFORMATION:

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Lys Ile Leu Arg Val Aan Pro Glu Gly Ser Val Lys Thr Val Phe
Gln Val Pro Glu Ile Val Aan Ays Ays Gys Gln Aan Gly Leu Leu
Gly Phe Ala Phe His Pro Aps Phe Lys Aan Aan Pro Tyr Ile Tyr Val
Ser Gly Thr Phe Lys Aan Pro Lys Thr Thr Asp Lys Glu Leu Pro Aan
Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Aan Lys Ala Thr Aps Thr Leu
Glu Lys Pro Val Aps Leu Leu Ala Gly Leu Pro Ser Ser Lys Ays His
Gln Ser Gly Arg Leu Val Ile Gly Pro Aps Gln Lys Ile Tyr Tyr Thr
Ile Gly Asp Gln Gly Arg Ays Gln Phe Ala Tyr Leu Phe Leu Pro Aan
Gln Ala Gln His Thr Pro Thr Gln Glu Leu Ser Gly Lys Aps Tyr
His Thr Tyr Met Gly Lys Val Leu Arg Leu Aan Leu Aps Gly Ser Ile
Pro Lys Aps Aan Pro Ser Phe Aan Gly Val Ile Ser His Ile Tyr Thr
Leu Gly His Arg Aan Pro Glu Gly Leu Ala Phe Thr Pro Aan Gly Lys
Leu Leu Gln Ser Glu Gln Gly Pro Aan Ser Aps Aps Glu Ile Aan Leu
Ile Val Lys Gly Gly Aan Tyr Gly Trp Pro Aan Val Ala Gly Tyr Lys
Aps Aps Ser Gly Tyr Ala Tyr Ala Ays Tyr Ser Ays Ala Ser Aan Lys
Aly Gln Ile Lys Aan Leu Glu Gln Aan Gly Leu Lys Val Ala Aan Gly
Val Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Aan Phe Val Pro
Pro Leu Lys Thr Leu Tyr Thr Val Gln Aps Thr Tyr Aan Aps
Pro Thr Cys Gly Asp Pro Thr Tyr Ile Cys Trp Pro Thr Val Thr Pro
Ser Ser Ala Tyr Val Tyr Lys Gly Gly Lys Aan Ile Ser Gly Trp
Glu Aan Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg
Ile Lys Leu Aps Pro Thr Tyr Ser Aps Thr Tyr Aps Aps Val Pro
Met Phe Lys Ser Aan Asn Arg Arg Asp Val Ile Ala Ser Pro Aps
Gly Aan Val Leu Tyr Val Leu Thr Thr Ser Gly Aan Val Glu Lys
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435 440 445

Lys Phe Thr Tyr Lys Ala Lys

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35 40 45

Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe

50 55 60

Gln Val Pro Glu Ile Val Asn Asp Ala Asp Gln Asn Gly Leu Leu

65 70 75 80

Gly Phe Ala Phe His Pro Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Val

85 90 95

Ser Gly Thr Phe Lys Asn Pro Lys Ser Thr Asp Lys Glu Leu Pro Asn

100 105 110

Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ala Thr Asp Thr Leu

115 120 125

Glu Lys Pro Val Asp Leu Leu Ala Gly Leu Pro Ser Ser Lys Asp His

130 135 140

Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Thr

145 150 155 160

Ile Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn

165 170 175

Gln Ala Gln His Thr Pro Thr Gln Gln Glu Leu Ser Gly Lys Asp Tyr

180 185 190

His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile

195 200 205

Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Ile Ser His Ile Tyr Thr

210 215 220

Leu Gly His Arg Asn Pro Gln Gly Leu Ala Phe Thr Pro Asn Gly Lys

225 230 235 240

Leu Leu Gln Ser Gly Asn Gln Gly Pro Asn Ser Asp Gln Ile Asn Leu

245 250 255

Ile Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys

260 265 270

Asp Asp Ser Gly Tyr Ala Tyr Ala Tyr Ser Ala Ser Asn Lys

275 280 285

 Ala Gin Ile Lys Asp Leu Asp Gin Asn Gly Leu Lys Val Ala Ala Gly

290 295 300
Val Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Asn Phe Val Pro 305 310 315 320
Pro Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asn Asp 325 330 335
Pro Thr Cys Gly Asp Met Thr Tyr Ile Cys Trp Pro Thr Val Thr Pro 340 345 350
Ser Ser Ala Tyr Val Tyr Gly Gly Lys Ala Ile Ser Gly Trp 355 360 365
Glu Asn Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg 370 375 380
Ile Lys Leu Asp Pro Thr Tyr Ser Ala Thr Tyr Asp Asp Ala Val Pro 385 390 395 400
Met Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp 405 410 415
Gly Asn Val Leu Tyr Val Leu Thr Asp Thr Ser Gly Asn Val Gln Lys 420 425 430
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Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe 50 55 60
Gln Val Pro Glu Ile Val Asn Asp Ala Asp Glu Gln Asn Gly Leu Leu 65 70 75 80
Gly Phe Ala Phe His Pro Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Val 85 90 95
Ser Gly Thr Phe Lys Asn Pro Lys Ser Thr Asp Lys Glu Leu Pro Asn 100 105 110
Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ala Thr Asp Thr Leu 115 120 125
Glu Lys Pro Val Asp Leu Val Ala Gly Leu Pro Ser Ser Lys Asp His 130 135 140
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1  5 10  15
Lys Phe Thr Tyr Lys Ala Lys
450 455

Phe Asp Lys Val Leu Leu Ser Asn Leu Asn Lys Pro His Ala Leu
19 25 30
Leu Trp Gly Pro Asp Asn Glu Ile Trp Leu Thr Glu Arg Ala Thr Gly
35 40 45
Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe
50 55 60
Gln Val Pro Glu Ile Val Asn Pro Asp Gly Glu Asn Asp Gly Leu Leu
65 70 75 80
Gly Phe Ala Phe His Pro Asp Phe Asn Leu Asn Pro Tyr Ile Tyr Val
85 90 95
Ser Gly Thr Phe Lys Asn Pro Ser Thr Asp Lys Glu Leu Pro Asn
100 105 110
Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ala Thr Asp Thr Leu
115 120 125
Glu Lys Pro Val Asp Leu Ala Gly Leu Pro Ser Ser Lys Asp His
130 135 140
Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Glu Gly Asp Ile Tyr Thr
145 150 155 160
Ile Gly Asp Gln Gly Arg Asn Glu Phe Ala Tyr Leu Phe Leu Pro Asn
165 170 175
Gln Ala Gln His Thr Pro Thr Gln Gln Glu Leu Ser Gly Lys Asp Tyr
180 185 190
His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile
195 200 205
Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Ile Ser His Ile Tyr Thr
210 215 220
Leu Gly His Arg Asn Pro Glu Gly Leu Ala Phe Thr Pro Asn Gly Lys
225 230 235 240
Leu Leu Gln Ser Glu Gly Pro Asn Ser Asp Gly Ile Asn Leu
245 250 255
Ile Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys
260 265 270
Asp Asp Ser Gly Tyr Ala Tyr Ala Asn Tyr Ser Ala Ala Ser Asn Lys
275 280 285

Ala Gln Ile Lys Asp Leu Glu Gln Asp Gly Leu Lys Val Ala Ala Gly
290 295 300

Val Pro Val Thr Lys Gly Ser Glu Trp Thr Gly Lys Aen Phe Val Pro
305 310 315 320

Pro Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Aen Aen Aen Pro
325 330 335

Pro Thr Cys Gly Asp Pro Thr Tyr Ile Asn Trp Pro Thr Val Ala Pro
340 345 350

Ser Ser Ala Tyr Val Tyr Lys Gly Gly Lys Ala Ile Ser Gly Trp
355 360 365

Glu Asn Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg
370 375 380

Ile Lys Leu Asp Pro Thr Tyr Ser Ala Thr Tyr Asp Ala Val Pro
385 390 395 400

Met Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp
405 410 415

Gly Asn Val Leu Tyr Val Leu Thr Ser Gly Aen Val Gln Lys
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Asp Asp Gly Ser Val Thr Asn Thr Leu Gln Asn Gly Ser Leu Ile
435 440 445

Lys Phe Thr Tyr Lys Ala Lys
450 455

SEQ ID NO 33
LENGTH: 455
TYPE: PRT
ORGANISM: Acinetobacter calcoaceticus
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NAME/KEY: MUTAGEN
LOCATION: (75)...
OTHER INFORMATION:

FEATURE:
NAME/KEY: MUTAGEN
LOCATION: (295)...
OTHER INFORMATION:

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LOCATION: (342)...
OTHER INFORMATION:

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NAME/KEY: MUTAGEN
LOCATION: (351)...
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SEQUENCE: 33

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35 40 45

Lys Ile Leu Arg Val Aen Pro Gly Ser Gly Ser Val Lys Thr Val Phe
50 55 60

Gln Val Pro Glu Ile Val Aen Asp Ala Asp Trp Gln Aen Gly Leu Leu
65 70 75 80

Gly Phe Ala Phe His Pro Asp Phe Lys Aen Aen Pro Tyr Ile Tyr Val
85 90 95

Ser Gly Thr Phe Lys Aen Pro Aen Ser Thr Asp Lys Glu Leu Pro Aen
Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ala Thr Asp Thr Leu
115 120 125
Glu Lys Pro Val Asp Leu Leu Ala Gly Leu Pro Ser Ser Lys Asp His
130 135 140
Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Glu Lys Ile Tyr Thr
145 150 155 160
Ile Gly Asp Glu Gly Arg Asn Glu Leu Ala Tyr Leu Phe Leu Pro Asn
165 170 175
Gln Ala Gln His Thr Pro Thr Gln Glu Leu Ser Gly Lys Asp Tyr
180 185 190
His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile
195 200 205
Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Ile Ser His Ile Tyr Thr
210 215 220
Leu Gly His Arg Asn Pro Glu Gly Ala Phe Thr Pro Asn Gly Lys
225 230 235 240
Leu Leu Gln Ser Glu Gln Pro Asn Ser Asp Asp Glu Ile Asn Leu
245 250 255
Ile Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys
260 265 270
Asp Asp Ser Gly Tyr Ala Tyr Ala Tyr Ser Ala Ser Asn Lys
275 280 285
Ala Gln Ile Lys Asp Leu Glu Gln Asn Gly Leu Val Ala Ala Gly
290 295 300
Val Pro Val Thr Lys Glu Ser Glu Thr Gly Lys Asn Phe Val Pro
305 310 315 320
Pro Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asn Asp
325 330 335
Pro Thr Cys Gly Asp Pro Thr Tyr Ile Cys Trp Pro Thr Val Thr Pro
340 345 350
Ser Ser Ala Tyr Val Tyr Lys Gly Gly Lys Ala Ile Ser Gly Trp
355 360 365
Glu Asn Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg
370 375 380
Ile Lys Leu Asp Pro Thr Tyr Ser Ala Tyr Asp Ala Val Pro
385 390 395 400
Met Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp
405 410 415
Gly Asn Val Leu Tyr Val Leu Thr Asp Thr Ser Gly Asn Val Glu Lys
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35   40    45
Lys Ile Leu Arg Val Asn Pro Gln Ser Gly Ser Val Lys Thr Val Phe
50   55    60
Gln Val Pro Glu Ile Val Asp Ala Asp Gly Gln Asn Gly Leu Leu
65   70    75    80
Gly Phe Ala Phe His Pro Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Val
85   90    95
Ser Gly Thr Phe Lys Asn Pro Tyr Ser Thr Asp Lys Glu Leu Pro Asn
100 105   110
Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ala Thr Asp Thr Leu
115 120   125
Glu Lys Pro Val Asp Leu Ala Gly Leu Pro Ser Ser Lys Asp His
130 135   140
Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Thr
145 150   155   160
Ile Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn
165 170   175
Gln Ala Gln His Thr Pro Thr Gln Gln Glu Leu Ser Gly Lys Asp Tyr
180 185   190
His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile
195 200   205
Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Ile Ser His Ile Tyr Thr
210 215   220
Leu Gly His Arg Asn Pro Gln Gly Leu Ala Phe Thr Pro Asn Gly Lys
225 230   235   240
Leu Leu Gln Ser Glu Gln Gly Pro Asn Ser Asp Asp Gly Ile Asn Leu
245 250   255
Ile Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys
260 265   270
Asp Asp Ser Gly Tyr Ala Tyr Asn Tyr Ser Ala Ala Ser Asn Lys
275 280   285
Ala Gln Ile Lys Asp Leu Gly Gln Asn Gly Leu Val Ala Ala Gly
290 295   300
Val Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Asn Phe Val Pro
305 310   315   320
Pro Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asp
325 330   335
Pro Thr Cys Gly Asp Pro Thr Tyr Ile Cys Trp Pro Thr Val Thr Pro
340 345   350
Ser Ser Ala Tyr Val Tyr Lys Gly Gly Lys Ala Ile Ser Gly Trp
355 360   365
Glu Asn Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg
370 375   380
Ile Lys Leu Asp Pro Thr Tyr Ser Ala Thr Tyr Asp Asp Ala Val Pro
385 390   395   400
What is claimed is:

1. A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising a polypeptide in which amino acids corresponding to residues of the following (1) and (2) in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are respectively substituted by other amino acids as compared with an amino acid sequence of the corresponding wild-type enzyme:

(1) an amino acid at position 295; and
(2) one or more amino acids selected from the group consisting of amino acids at positions 75, 168, 169, 342, 347 and 351.

2. A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising any of the following amino acid sequences (1) to (6):

(1) a polypeptide in which amino acids corresponding to residues at positions 169, 295, 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;
(2) an amino acid sequence in which amino acids corresponding to amino acids at positions 295 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;
(3) an amino acid sequence in which amino acids corresponding to amino acids at positions 295, 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;
(4) an amino acid sequence in which amino acids corresponding to amino acids at positions 169, 295 and 342 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;
(5) an amino acid sequence in which amino acids corresponding to amino acids at positions 75, 295, 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme;
(6) an amino acid sequence in which amino acids corresponding to amino acids at positions 342 and 351 in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are respectively substituted by other amino acids, as compared with an amino acid sequence of the corresponding wild-type enzyme.

3. A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising any of the following amino acid sequences (1) to (6):

(1) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 169, 295, 342 and 351 are substituted by other amino acids respectively;
(2) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 295 and 351 are substituted by other amino acids respectively;
(3) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 295, 342, and 351 are substituted by other amino acids respectively;
(4) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 169, 295 and 342 are substituted by other amino acids respectively;
(5) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 75, 295, 342 and 351 are substituted by other amino acids respectively; and
(6) an amino acid sequence of SEQ ID NO: 1 in which amino acids at positions 342 and 351 are substituted by other amino acids respectively.

4. A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising any of amino acid sequences of SEQ ID NOs: 28 to 34.

5. A modified pyrroloquinoline quinone-dependent glucose dehydrogenase, comprising a polypeptide in which amino acids corresponding to residues at position 295 and one or more amino acids selected from the group consisting of amino acids at positions 75, 169, 342 and 351, wherein at least one amino acid must be an amino acid at position 351, in pyrroloquinoline quinone-dependent glucose dehydrogenase derived from *Acinetobacter calcoaceticus* are substituted by amino acids of the following (i)-(v) as compared with an amino acid sequence of the corresponding PQQGDHs derived from *Genus Acinetobacter*:

(i) Glu, Asp, Tyr, Phe, Cys or Val substitute an amino acid at position 295;
(ii) Trp substitutes an amino acid at position 75;
(iii) Phe substitutes an amino acid at position 169;
(iv) Pro substitutes an amino acid at position 342;
(v) Thr substitutes an amino acid at position 351.

* * * * *